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PRINCIPAL INVESTIGATOR: R. Martin Roop, Ph.D.

CONTRACTING ORGANIZATION: Louisiana State University
Shreveport, Louisiana 71130-3932

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6. AUTHOR(S)
R. Martin Roop, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Louisiana State University Health Sciences Center,

Shreveport, Louisiana 71130-3932

E-MAIL:
rroop@lsuhsc.edu

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Results to date from the studies funded under this contract suggest that *Brucella* genes involved in maintaining efficient stationary phase physiology and allowing the brucellae to make effective use of the nutrients available in the phagosomal compartment make good targets for the construction of mutants with defined levels of attenuation which may prove useful as vaccine candidates. For example, the well characterized antioxidants KatE and SodC protect *B. abortus* from reactive oxygen intermediate (ROI)-mediated killing in *in vitro* assays, but experiments employing *katE* and *sodC* mutants indicate that these antioxidants are probably more important for protecting the intracellular brucellae from the endogenous ROIs generated by stationary phase metabolism than they are for detoxifying the products of the oxidative burst of host macrophages. The brucellae also experience significant nutrient deprivation in the phagosomal compartment, and preliminary results suggest that the *bacA* gene product functions in iron acquisition in this environment. Tn*5* mutants of *B. abortus* which display nutritional defects *in vitro* and are unable to survive and replicate in cultured murine macrophages have also been identified and are presently being characterized.

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INTRODUCTION

Brucellosis is a serious zoonotic disease (Acha and Szyfres, 1980) which poses a potential threat to military personnel stationed in many areas of the world. These individuals are at risk either through accidental exposure to infected animals or animal products (Nicoletti, 1989) or through deliberate exposure to *Brucella* spp. as a component of biological warfare by an opposing military force (Huxsoll et al., 1987; Kaufmann et al., 1997). Unfortunately, there is currently no safe, effective brucellosis vaccine for use in humans. Prolonged survival and replication in host macrophages is a key component of disease production by *Brucella* spp. (Baldwin and Winter, 1994), and current evidence indicates that reactive oxygen intermediate (ROI)-mediated killing is the primary mechanism by which host macrophages eliminate intracellular brucellae (Jiang et al., 1993). Therefore, cellular components which contribute to the resistance of the brucellae to oxidative killing by host phagocytes represent important virulence determinants. Biochemical and genetic studies have clearly shown that bacterial stress response proteases of the high temperature requirement A (HtrA) family have the capacity to degrade oxidatively damaged proteins before they accumulate to toxic levels within cells (Davies and Lin, 1988; Johnson et al., 1991; Li et al., 1996). In performing this function, members of this particular class of periplasmic serine proteases serve as an important secondary line of defense against ROI-mediated killing. Genetic studies in numerous bacteria including *Brucella abortus* and *Brucella melitensis* have confirmed the participation of the HtrA protease in cellular defense against oxidative damage (Elzer et al., 1994; Phillips et al., 1995; Robertson et al. 1996), and further suggest that this protease contributes to the survival and replication of the brucellae in host macrophages (Elzer et al., 1996a) and their virulence in natural and experimental hosts (Edmonds et al., 1997, Elzer et al., 1994; Elzer et al. 1996a; Elzer et al. 1996b, Phillips et al. 1995; Phillips et al. 1997, Robertson et al. 1996). *Salmonella* strains carrying *htrA* mutations have been used successfully as vaccines in both mice (Chatfield et al., 1992) and humans (Tacket et al., 1997), and evidence recently obtained with pregnant goats suggests that *Brucella htrA* mutants also hold promise as vaccine candidates (Phillips et al., 1997). Unfortunately, the attenuation of the *Brucella htrA* mutants observed in the original studies in mice was limited to the early stages of infection (Elzer et al. 1996a; Phillips et al. 1995), and this residual virulence prevented their evaluation as experimental vaccines in this important model of human brucellosis. Therefore, the original objective of the proposed studies outlined in the Statement of Work for contract DAMD17-98-C-8045 was to introduce secondary mutations into *B. abortus* and *B. melitensis htrA* mutants which would enhance and stabilize the attenuation of these strains in BALB/c mice.

As detailed in last year's annual report, soon after the initiation of this project is was found that the *Brucella* mutants used in the original studies were in fact *htrA cycL* double mutants, the secondary *cycL* mutation arising from the close proximity of this gene to the 5' end of the *Brucella htrA* and the strategy employed for gene disruption (Phillips et al., 1999). Subsequent analysis of an authentic *B. abortus htrA* mutant in mice revealed no significant attenuation for this strain. Moreover, introduction of selected tertiary mutations into the *htrA cycL* mutants did not enhance the attenuation of these strains in mice beyond their original level (Kovach and Roop, 1996). For these reasons, a revised Statement of Work was submitted for this contract and approved. The new objectives of this project focus primarily on exploring the potential of *B. abortus* and *B. melitensis hfq* and *bacA* mutants as vaccine candidates in the mouse model and further characterizing the interactions of these highly attenuated mutants with cultured murine macrophages in an effort to gain a better understanding of the basis for their attenuation. *Brucella katE* (Sha et al., 1994) and *sodC* (Bricker et al., 1990) mutants will also be used to evaluate the contribution of the corresponding gene products to the well characterized resistance of the brucellae to the oxidative killing pathways of host phagocytes (Baldwin and Roop, 1999). Although experimental evidence clearly establishes a generalized defect in stationary phase physiology as the basis for the extreme and stable attenuation of *Brucella hfq* mutants (Robertson and Roop, 1999), the biologic function of the *bacA* gene product is presently unknown (LeVier et al., 2000). Thus, another focus of this project is to thoroughly evaluate the

phenotypic characteristics of the *B. abortus bacA* mutant KL7 in an attempt to define the biological function of the corresponding gene product.

BODY

I. *B. abortus katE* and *sodC* mutants are attenuated in the BALB/c mouse model but display wild type survival and replication in cultured murine macrophages.

Based on our previous observation that the *B. abortus katE* (MEK6) and *sodC* (MEK2) mutants displayed significant attenuation in BALB/c mice, we evaluated the capacity of these strains to survive and replicate in cultured murine resident peritoneal macrophages following opsonization with *Brucella*-specific hyperimmune mouse serum. In multiple experiments, both of these strains showed intracellular survival and replication profiles in macrophages equivalent to those of the parental strain 2308 (Figure 1). Furthermore, treatment of the cultured macrophages with IFN-γ did not alter the intracellular replication profiles of MEK2 and MEK6 relative to that of 2308 (data not shown). As opsonization of the brucellae with *Brucella*-specific IgG and IFN-γ activation of the macrophages would be expected to maximize the oxidative killing capacity of these phagocytes, these findings strongly suggest that the basis for the attenuation of the *B. abortus katE* and sodC mutants is not a defect in their ability to resist the oxidative burst of host macrophages.

In an attempt to confirm the link between the *katE* mutation and the attenuation of MEK6 in BALB/c mice, a wild type copy of the *Brucella katE* was cloned into pBBR1MMCS and the resulting plasmid (pMEK21) introduced into this mutant. Introduction of pMEK21 into MEK6 resulted in the production of catalase activity by this strain as evidenced by the production of bubbles when colonies were exposed to H_2O_2, and restored the capacity of MEK6 to withstand exposure to H_2O_2 in disk assays to levels comparable to that of 2308 (Figure 2). When MEK6 (pMEK21) was evaluated in BALB/c mice, however, this strain displayed a spleen colonization profile through 12 weeks post infection identical to that of MEK6. Evaluation of reisolates of MEK6 (pMEK21) for chloramphenicol resistance revealed that this strain had lost the *katE*-bearing plasmid at some point after 8 weeks post infection. Similar plasmid stability problems have been encountered in our attempts to complement the phenotype of the *B. abortus sodC* mutant MEK2 with a cloned copy of the *sodC* gene. We are presently constructing pGL10-based plasmids carrying *katE* and *sodC* and will use these plasmids in future attempts to complement the phenotypes of the *B. abortus katE* and *sodC* mutants. pGL10 is an RK2-based plasmid vector which is maintained at low copy number in *Brucella* (2-4 copies per cell; Robertson et al., 2000), which should reduce the potential for problems associated with overproduction of KatE or SodC. This plasmid also contains an active partitioning system which should enhance its maintenance during *in vivo* replication of the brucellae.

II. *In vitro* and *in vivo* characterization of the *B. abortus bacA* mutant KL7 and the *B. melitensis bacA* mutant KL20.

Previous studies in our laboratory have established that the *B. abortus bacA* mutant KL7 shows significant attenuation in BALB/c mice and defective replication in cultured murine macrophages (LeVier et al, 2000). Our attempts to complement this phenotype with a plasmid borne copy of the *bacA* gene, however, were unsuccessful. In fact, as noted in last year's annual report for this contract, overexpression of *bacA* appeared to enhance the attenuation of KL7 in mice and in cultured macrophages. To remedy this situation, we employed a *sacB* containing vector to introduce a wild type copy of the *bacA* gene onto the chromosome of KL7, producing a merodiploid state in which both the wild type *bacA* as well as the disrupted *bacA* allele were present on the chromosome separated by the *sacB*-containing vector. Growth of the resulting strains in the presence of sucrose was then used to force the resolution of the merodiploid state, and ampicillin sensitive (loss of the vector) and kanamycin sensitive (loss of the mutated *bacA* allele) colonies selected. Using this strategy, the mutant *bacA* allele in KL7 was replaced with the wild type *bacA*, resulting in the construction of KL74.3. The genotype of this strain was confirmed by Southern blot analysis with *bacA*-, kanamycin resistance gene-, and vector-specific probes, and KL74.3 displayed an *in vitro* sensitivity to bleomycin equivalent to that of

2308 (data not shown). More importantly, KL74.3 demonstrated a spleen colonization profile in BALB/c mice (Figure 3) and intracellular survival and replication in cultured murine macrophages (Figure 4) identical to that of 2308. These findings verified the link between the *bacA* mutation in KL7 and the dramatic attenuation of this strain in mice.

In order to get a better idea of the basis for the attenuation of KL7 in mice, we have begun to evaluate the intracellular survival and replication of this strain in cultured murine macrophages more closely. Specifically, the effects of opsonization and IFN-γ activation on the intracellular survival of KL7 in cultured macrophages is being examined. Although these studies are ongoing, our preliminary findings indicate that KL7 is defective in its capacity to replicate in cultured murine macrophages regardless of their state of activation (data not shown) or the mode of entry of the brucellae into these phagocytes (Figure 5).

Although the *B. abortus bacA mutant* shows significant and stable attenuation in the BALB/c model, the biological function of the *bacA* gene product is presently unknown (LeVier et al., 2000). In an effort to define the function of BacA and therefore gain insight into the basis for the attenuation of the *B. abortus bacA* mutant, we have begun to examine KL7 *in vitro* for phenotypic characteristics that might yield some clue with regard to the function of the corresponding gene product. Our initial findings from these studies strongly suggest that BacA is involved in the transport of Fe^{++} into the bacterial cell. For example, KL7 is much more sensitive to the chelator EDDA than 2308; indeed, KL7 displays sensitivity to EDDA at the same level as does the *ent* mutant BHB1, a derivative of 2308 that cannot produce the siderophore 2,3-dihydroxybenzoic acid (Bellaire et al, 1999). Moreover, when 2308 and KL7 are grown in an iron-deprived minimal medium, both strains show equivalent growth restriction compared to growth in iron-replete medium. When these iron-starved cultures are supplemented with $FeSO_4$, however, 2308 shows enhanced growth, while KL7 does not. Other distinctive *in vitro* phenotypic characteristics of KL7 include an increased sensitivity to NaCl and the antibiotic puromycin (data not shown) compared to the parental strain 2308. Growth curves of KL7, KL74.3 and 2308 in brucella broth indicate that KL7 has a prolonged lag phase and slower growth rate in this complex medium compared to these two other strains (Figure 6). Interestingly, this *in vitro* growth defect is not observed when KL7 is grown on a solid medium nor does it affect the capacity of this mutant to establish spleen and liver infections in BALB/c mice identical to those produced by 2308 and KL74.3 through 3 weeks post infection (Figure 3).

When the *B. melitensis bacA* mutant KL20 was evaluated in BALB/c mice, this strain was found to be much less attenuated than its *B. abortus* counterpart (Figure 7). This result was not a total surprise, however, as in related studies, the *B. melitensis bacA* mutant displayed a level of virulence equivalent to that of 16M in goats infected during the third trimester of pregnancy (Roop et al., 2000). These findings demonstrate that the phenotype imparted by the *bacA* mutation in *Brucella* strains can differ greatly based on the genetic background into which it is introduced. One possible basis for this difference might be related to iron acquisition as will be discussed in the Conclusions section.

III. Evaluation of the capacity of the *B. abortus bacA* mutant KL7 to elicit protective immunity in BALB/c against challenge with virulent *B. abortus* 2308.

An experiment has been designed and will be carried out in the next few weeks which will test the potential of KL7 to induce protective immunity in mice. *B. abortus* S19 will serve both as the positive control for these studies as well as a basis for comparison with regard to the degree of protective immunity provided by KL7.

IV. Construction of *B. abortus* Tn*5* mutants demonstrating nutritional defects *in vitro* and defective survival and replication in cultured murine macrophages.

Experimental evidence suggests that the brucellae encounter a considerable degree of nutrient deprivation during their long term residence in host macrophages (Crawford et al., 1996; Robertson and Roop, 1999). Correspondingly, the ability of these organisms to maintain their metabolic versatility is likely critical to their capacity to survive for prolonged periods in the phagosomal compartment and produce disease in the host. To test this hypothesis, *B. abortus* 2308 was subjected to Tn*5* mutagenesis and the resulting transconjugants screened for nutritional

defects by replica plating on blood agar and minimal medium plates. Five Tn*5* mutants have been identified to date which demonstrate growth comparable to that of 2308 on blood agar but unlike 2308 are unable to grow on the minimal medium plates. Three of these mutants have subsequently been examined for their survival and replication in cultured murine macrophages, and all three show defective intracellular survival compared to 2308 (Figure 8). In upcoming months, the spleen colonization profiles of these mutants in BALB/c mice will be compared to that of 2308 and the Tn*5*-disrupted loci in these strains cloned and characterized.

V. Functional complementation of a *Salmonella typhimurium hfq* mutant by the cloned *B. abortus hfq* and discovery of a putative *rpoS* homolog in *Sinorhizobium meliloti*.

Previous studies have shown that the *B. abortus hfq* gene product is required for the maintenance of stationary phase physiology (Robertson and Roop, 1999). Moreover, *Brucella hfq* mutants show dramatic and stable attenuation in both experimental and natural hosts (Roop et al., 2000), suggesting that the capacity to establish and maintain stationary phase physiology is essential for the long term residence of the brucellae in host macrophages. Introduction of a plasmid carrying the cloned *B. abortus hfq* gene into a *S. typhimurium hfq* mutant carrying a *katE*::*lacZ* fusion restores the expression of this gene fusion. Because *katE* expression in *Salmonella* requires the presence of a functional RpoS, this finding indicates that the *B. abortus* HF-I facilitates proper translation of the *rpoS* mRNA in *S. typhimurium*. This supports our hypothesis that the *Brucella hfq* gene product regulates stationary phase gene expression in a manner similar to that of its *E. coli* and *S. typhimurium* counterparts (Muffler et al., 1997). In further support of our proposal, a search of the *Sinorhizobium meliloti* genome sequence in the NCBI database has yielded what we believe is a gene encoding an RpoS homolog. More importantly, because the global stationary phase defect observed in the *B. abortus hfq* mutant Hfq3 resembles that described for bacterial *rpoS* mutants, we suspect that *B. abortus* possesses an RpoS homolog. We are presently trying to use the putative RpoS encoding sequences from the *S. meliloti* genome to identify an *rpoS* homolog in the genome of *B. abortus* 2308.

VI. Attempts to construct a *B. abortus hfq bacA* mutant have been unsuccessful.

The dramatic and stable attenuation of the *B. abortus hfq* and *bacA* mutants in mice suggests that these strains hold promise as potential vaccine candidates. In this regard, if the physiologic defects which underlie the attenuation of these strains are independent, then combining the *bacA* and *hfq* mutations into a single strain may represent an effective and reliable means of stabilizing the attenuation of a live vaccine strain in the host. In our attempts to construct a *B. abortus hfq bacA* mutant, we have repeatedly isolated transformants with antibiotic resistance and Southern blot analysis profiles indicative of single crossover integration of the *bacA* mutation onto the chromosome producing a merodiploid containing both the wild type and mutated forms of *bacA*. In contrast, we have been unable to isolate transformants with phenotypic and genotypic properties indicative of replacement of the wild type *bacA* allele with the mutated form in the *hfq* background. These findings suggest that the *hfq* and *bacA* mutations cannot be combined in *B. abortus* 2308. In future studies, we will employ a *sacB*-based gene replacement strategy to definitively determine whether or not these two mutations can be combined in the *B. abortus* 2308 or *B. melitensis* 16M genetic backgrounds.

VII. Spleen colonization profiles of *B. abortus* 2308 in NADPH oxidase knockout mice

Considerable experimental evidence indicates that the oxidative killing pathways of host macrophages are critical for the control of intracellular brucellae (Baldwin and Roop, 1999). Consequently, we have recently begun to use C57BL6 NADPH oxidase knockout (*cybb*) mice and their wild type counterparts to examine this relationship further. In preliminary studies, NADPH oxidase knockout mice appear to be better able to control spleen infection with *B. abortus* 2308 than the parental strain of mice at one week post infection (Figure 9). At four weeks post infection, however, greater numbers of brucellae were recovered from the spleens of NADPH oxidase knockout mice infected with 2308 than were recovered from the spleens of wild type C57BL6 mice infected with this strain. When resident peritoneal macrophages from both sets of mice were evaluated for their capacity to control the intracellular replication of *B. abortus* 2308, identical results were obtained with macrophages obtained from the NADPH

oxidase knockout and wild type C57BL6 mice (data not shown). Although these studies are preliminary in nature, our findings to date are consistent with the oxidative burst of host macrophages being of minor importance in the control of *Brucella* infections during the very early stages of the disease, but taking on increasing importance as the infection progresses toward the chronic state.

VIII. Attempts to develop an *in vivo* expression technology (IVET) system for detecting *Brucella* genes exclusively expressed in the host based on the essential nature of the *asd* gene have been unsuccessful.

The product of the *asd* gene, aspartate β-semialdehyde dehydrogenase, is a critical component of the diaminopimelic acid (DAP) biosynthesis pathway. DAP, in turn is an essential component of the peptidoglycan of bacterial cell walls. In the majority of the bacteria that have been studied to date, *asd* represents an essential gene, and correspondingly *asd* mutants can only be constructed if they are supplied with exogenous DAP in the growth medium. The essential nature of *asd* has been exploited in *Salmonella* to construct a positive selection system for plasmid vectors, and also utilized as a means of ensuring stable expression of heterologous genes in live recombinant *Salmonella* vaccine strains (Galan et al., 1990). In a similar fashion, the essential nature of the *asd* gene makes it attractive as the basis for the construction of an IVET system designed to identify *Brucella* promoters which are only active during *in vivo* growth.

The *asd* gene from *B. abortus* 2308 was cloned by virtue of its ability to restore the capacity of the *E. coli asd* mutant χ6097 to grow in the absence of exogenous DAP. The identity of the cloned *B. abortus asd* gene was confirmed by nucleotide sequence determination. An ampicillin resistance gene was inserted into the coding region of the cloned *asd* gene and the lack of function of the resulting allele was verified by its inability to complement the *asd* mutation in χ6097. Multiple attempts to replace the *B. abortus* 2308 *asd* with this mutant allele were unsuccessful, although transformants carrying both the wild type *asd* and disrupted *asd* were isolated routinely. Upon learning that similar difficulties had been encountered by investigators at the Walter Reed Institute of Army Research during their attempts to construct a *B. melitensis asd* mutant, we have decided for the present to pursue other essential genes as the basis for the design of IVET strategies for *B. abortus*.

IX. Construction and initial characterization of a *B. abortus ctpA* (*prc*) mutant.

The *prc* gene encodes a periplasmic stress response protease which has many properties similar to those of the HtrA protease (Hara et al., 1991). Most important, the *prc* gene product is a member of the class of stress response proteases which have the capacity to degrade oxidatively damaged proteins (Davies and Lin, 1988), and studies with *prc* mutants of *S. typhimurium* indicate that these strains are defective in their capacity to survive and replicate in host macrophages (Bäumler et al., 1994). One of the original objectives of the research project funded by this contract was to determine if combining the *prc* and *htrA* mutations in *B. abortus* 2308 and *B. melitensis* 16M would result in the construction of mutant strains with sufficient attenuation to make them useful as vaccine candidates. Such studies would also provide a genetic test of the hypothesis that the *prc* and *htrA* gene products perform overlapping biological functions.

The *B. abortus prc* homolog was recently cloned in the laboratory of Dr. Shirley Halling at the National Animal Disease Center (Halling and Koster, 1999). It was given the designation *ctpA* based on its extensive homology with the *ctpA* gene of *Bartonella bacilliformis* (Mitchell and Minnick, 1997). We obtained a cloned copy of the *B. abortus ctpA* from Dr. Halling, and constructed a mutant *ctpA* allele containing a chloramphenicol resistance gene disrupting the *ctpA* coding region. A previously described gene replacement strategy (Elzer et al., 1994) was used to replace the wild type *ctpA* in the *B. abortus* 2308 chromosome with the disrupted *ctpA*, resulting in the construction of *B. abortus* VKG12. In contrast to the *B. abortus htrA* mutant RWP11, the *ctpA* mutant shows no significant difference from 2308 with respect to its sensitivity to H_2O_2 or puromycin in disk sensitivity assays (data not shown). In fact, *in vitro* analysis of VKG12 has to date revealed no distinctive phenotypic characteristics for this mutant. When the

B. abortus ctpA mutant was evaluated in cultured murine macrophages, however, this strain showed a significant defect in its ability to survive and replicate in these phagocytes compared to *B. abortus* 2308 (Figure 10). Further *in vitro* evaluation of VKG12 will be required to define the biological function of the *B. abortus ctpA* gene product and its potential contribution to virulence. It is also notable that we have repeatedly been unable to introduce the *ctpA* mutation into the *B. abortus htrA* mutant RWP11 using standard gene replacement strategies. One plausible explanation for this finding is that HtrA and CtpA perform overlapping biological functions and that the presence of at least one of these proteases is required for cell viability. A more stringent and carefully controlled examination of the compatibility of the *htrA* and *ctpA* mutations in *B. abortus* employing a *sacB* based gene replacement strategy will be necessary, however, before such a relationship between the corresponding gene products can be verified.

KEY RESEARCH ACCOMPLISHMENTS

- Discovering that the *katE* and *sodC* gene products are probably more important for protecting the intracellular brucellae from endogenous ROIs generated by the maintenance metabolism associated with stationary phase physiology than they are for detoxifying the products of the oxidative burst of host macrophages.

- Determining that the *Brucella* BacA may play a role in iron acquisition, and finding that the *bacA* mutation imparts a much less dramatic phenotype in terms of attenuation on *B. melitensis* 16M than it does on *B. abortus* 2308.

- Identification of *B. abortus* Tn*5* mutants with nutritional defect *in vitro* which show defective survival and replication in cultured murine macrophages.

- Establishing that C57BL6 NADPH oxidase knockout (*cybb*) mice and phagocytes obtained from these animals are useful tools for better defining the role of the oxidative killing pathways of host phagocytes in protective immunity against brucellosis.

REPORTABLE OUTCOMES

Manuscripts, abstracts and presentations:

Robertson, G. T., and R. M. Roop II. 1999. The *Brucella abortus* host factor I (HF-I) protein contributes to stress resistance during stationary phase and is a major determinant of virulence in mice. Mol. Microbiol. 34:690-700 - **manuscript**

Robertson, G. T., M. E. Kovach, C. A. Allen, T. A. Ficht, and R. M. Roop II. 2000. The *Brucella abortus* Lon functions as a generalized stress response protease and is required for wild-type virulence in BALB/c mice Mol. Microbiol. 35:577-588 - **manuscript**

LeVier, K., R. W. Phillips, V. K. Grippe, R. M. Roop II, and G. C. Walker. 2000. Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. Science 287:2492-2493 - **manuscript**

Parent, M, K. LeVier, G. Walker, G. Robertson, B. Bellaire, R. M. Roop II, and C. Baldwin. 1999. Assessing the pathogenesis of several *Brucella abortus* 2308 mutants in IFN-γ knock out mice. Proc. 80th Annu. Conf. Res. Work. Anim. Dis., Abstr. 10. - **abstract**

LeVier, K., R. W. Phillips, V. K. Grippe, G. C. Walker, and R. M. Roop II. "Identification of a developmental gene in *Brucella* and genetic evidence for its role in virulence in the mammalian host." Annual Meeting of the South Central Branch of the American Society for Microbiology, New Orleans, LA., October 1999. - **presentation**

G. T. Robertson, J. Gee, S. Köhler, and R. M. Roop II. "Synthetic enhancement of phenotype: evidence for overlapping function of the *Brucella* abortus Lon and ClpAP proteases in pathogenesis." Annual Meeting of the South Central Branch of the American Society for Microbiology, New Orleans, LA., October 1999 - **presentation**

G. T. Robertson, and R. M. Roop II. "Maintenance of stationary phase is required for establishment of chronic infection by *Brucella abortus* in the murine model." Annual Meeting of the South Central Branch of the American Society for Microbiology, New Orleans, LA., October 1999 - **presentation**

R. W. Phillips, and R. M. Roop II. "A reevaluation of the biological function of the *Brucella abortus* HtrA protease." 52nd Annual Brucellosis Research Conference, Chicago, IL., November, 1999 - **presentation**

Grippe, V. K., M. E. Kovach, R. W. Phillips, and R. M. Roop II. "A genetic analysis of the roles of *Brucella* antioxidants in virulence in mice." 52nd Annual Brucellosis Research Conference, Chicago, IL., November, 1999 - **presentation**

Robertson, G. T., J. Gee, S. Köhler, and R. M Roop II. "Synthetic enhancement of phenotype: evidence for overlapping biological function of the *Brucella abortus* Lon and ClpAP proteases." 52nd Annual Brucellosis Research Conference, Chicago, IL., November, 1999 - **presentation**

LeVier, K., R. W. Phillips, V. K. Grippe, R. M. Roop II, and G. C. Walker. "A plant symbiont and a mammalian pathogen require a common gene product for survival inside eukaryotic cells." 52nd Annual Brucellosis Research Conference, Chicago, IL., November, 1999 - **presentation**

Grippe, V. K., M. E. Kovach, R. W. Phillips, and R. M. Roop II. "The *Brucella katE* and *sodC* gene products contribute to the maintenance of chronic infection in mice." 44th Annual Meeting of the Wind River Conference on Procaryotic Biology, Estes Park, CO., June, 2000 – **presentation**

Gee, J. M., G. T. Robertson, S. Köhler, and R. M. Roop II. "Synthetic enhancement of phenotype: evidence for overlapping biological function of the *Brucella abortus* Lon and ClpAP proteases." 44th Annual Meeting of the Wind River Conference on Procaryotic Biology, Estes Park, CO., June, 2000 - **presentation**

R. M. Roop II. "Practical benefits from molecular-based studies of the pathogenesis of *Brucella* infections." Joy Goodwin Lecture, Auburn University College of Veterinary Medicine. January 27, 2000 - **presentation**

R. M. Roop II. "Brucellosis – a case of symbiosis gone bad?" Department of Microbiology and Immunology Seminar Series, Tulane University Medical School, February 23, 2000 - **presentation**

R. M. Roop II. "*Brucella* stationary phase gene expression and virulence." Division of Basic Biomedical Sciences Seminar Series, University of South Dakota School of Medicine, April 7, 2000 - **presentation**

R. M. Roop II. "Brucellosis – a case of symbiosis gone bad?". Session 154/Z, Molecular Biology of Animal Pathogens, 100th Annual Meeting of the American Society or Microbiology, Los Angeles, CA, May 23, 2000 – **presentation (invited speaker)**

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Employment or research opportunities applied for and/or received based on experiences/training supported by this award:
Gregory T. Robertson – Postdoctoral Fellow, Infectious Disease Research Laboratories, Lilly Research Laboratories, February 2000

Vanessa K. Grippe – Research Associate, National Institutes of Health, scheduled to start in October 2000

CONCLUSIONS

I. The *Brucella* KatE and SodC appear to be more important for cellular defense against the endogenous ROIs generated during the maintenance metabolism associated with stationary phase physiology than they are for detoxifying the products of the oxidative burst of host macrophages.

A considerable amount of experimental evidence indicates that the oxidative killing pathways of host phagocytes represent a critical component of host immunity against *Brucella* infections (Baldwin and Roop, 1999). Correspondingly, *Brucella* spp. in general display an unusually high level of endogenous resistance to killing by ROIs such as H_2O_2 and O_2^- *in vitro* compared to other bacteria, and studies employing *katE* and *sodC* mutants indicate that the corresponding gene products contribute significantly to the innate resistance of the brucellae to oxidative killing (Grippe et al., 1999). Based on such findings, it has been proposed that the *Brucella katE* and *sodC* are important in vivo for protection against the oxidative killing pathways of host phagocytes (Tatum et al., 1992; Sha 1994; Grippe et al., 1999). Although more studies still need to be performed, our initial findings with the *B. abortus katE* and *sodC* mutants suggest that the corresponding gene products are not required in order for the brucellae to survive and replicate efficiently in host macrophages during the early stages of infection in the mouse model. In fact, as detailed in last year's annual report significant attenuation of neither mutant is seen until the latter stages of infection, well after the plateau phase has been established and maintained for several weeks. In a similar fashion, evaluation of these mutants in cultured murine macrophages showed that they survive and replicate in these phagocytes with essentially the same efficiency as virulent strain 2308 at both 24 and 48 hours post infection. Since previous studies have shown that the oxidative killing mechanisms of cultured murine macrophages have the greatest effective during the first several hours following entry of the brucellae (Jiang et al., 1993), these finding argue against a major role for KatE and SodC in resistance to these oxidative killing mechanisms. The recent finding that a *B. melitensis katE* mutant shows virulence in pregnant goats equivalent to the virulent parental strain 16M (Roop et al., 2000) also argues against a major role for KatE as a defense mechanism against the oxidative killing pathways of host macrophages.

One of the characteristic features of bacteria as they enter stationary phase physiology is the increased expression of genes encoding oxidative defense mechanisms (Hengge-Aronis, 1996). For example the *E. coli* and *S. typhimurium katE* and *sodC* show stationary phase-specific induction. Experimental evidence also suggests that expression of the *Brucella katE* (G. Robertson and J. Gee, unpublished) and *sodC* (R. Vemulapalli, person communication) increases as these organisms enter stationary phase. One possible explanation for this global induction of genes involved in protection against oxidative damage is that the corresponding gene products are necessary to protect the bacterium against the ROIs generated as the result of the maintenance metabolism associated with stationary phase physiology. Bacteria which rely upon a respiratory metabolism are prone to the endogenous accumulation of ROIs due to incomplete reduction of O_2 when their metabolism slows down, as would be expected during a period when bacterial cells are experiencing little net replication but instead diverting their metabolism to maintaining cell viability. Thus, one plausible explanation for the accelerated clearance of the *B. abortus katE* and *sodC* mutants occurring late in plateau phase is that eventually the endogenous ROIs accumulating in the stationary phase brucellae reach a point that the other ROI defenses in the cell cannot deal with them and they become toxic. If fact, it may actually be that the buildup of toxic endogenous ROIs in the brucellae combined with the presence of *Brucella*-specific IgG and activated macrophages in the host during these stages of the infection may serve to overwhelm the *B. abortus katE* and *sodC* mutants during the late stages of infection in the mouse model. Further studies with *Brucella* strains carrying defined mutations affecting their oxidative defense mechanisms and transgenic mouse strains with defined mutations affecting the ability of their phagocytes to generate an oxidative burst will be required to gain a better understanding of the role of oxidative killing by host macrophages in protective immunity to brucellosis.

II. The *Brucella* BacA may be involved in the transport of iron.

Although the *Sinorhizobium meliloti bacA* gene product is absolutely required for the transition of this bacterial endosymbiont from the free-living form to the non-replicating intracellular form that fixes nitrogen in the host plant (Glazebrook et al., 1993), and the homologous gene in *B. abortus* is essential for maintenance of chronic infection in the BALB/c mouse model (LeVier et al., 2000), the biological function of BacA in neither bacterium is known. For this reason, we have begun to evaluate the *B. abortus bacA* mutant KL7 for phenotypic characteristics *in vitro* that might provide some insight into the physiologic function of the corresponding gene product. In conjunction with these studies, Gail Ferguson, a postdoctoral fellow in Dr. Graham Walker's laboratory at MIT, is thoroughly evaluating the *in vitro* characteristics of a *S. meliloti bacA* mutant. To date, the results of these phenotypic studies suggest that BacA may be a non-specific transporter of a number of divalent cations including Ca^{++} and Fe^{++} which resides in the cytoplasmic membrane. Although the definitive experiments to establish this function have not been performed as yet, such a function could explain the differences in virulence in mice observed for the *B. abortus bacA* mutant KL7 and the *B. melitensis bacA* mutant KL20. Following construction of a *B. melitensis* mutant (BHB10) which is deficient in the production of the siderophore 2,3-dihydroxybenzoic acid (DHBA), one of the interesting characteristics that was noted for this mutant was that while no siderophore activity was detected for this strain in the catechol siderophore specific Arnow assay, residual siderophore activity could be detected in the CAS assay, which detects siderophore activity regardless of the chemical nature of the iron-binding compound (B. Bellaire, unpublished). This is in contrast to the corresponding *B. abortus ent* mutant BHB1 for which siderophore activity cannot be detected in either assay (Bellaire et al. 1999). These findings indicate that *B. melitensis* 16M possesses an iron acquisition system that is not present in *B. abortus* 2308. With this in mind, one could envision that BacA provides the brucellae with iron (likely Fe^{++}) in the phagosomal compartment of host macrophages. When BacA function is lost, *B. abortus* cannot obtain sufficient iron to maintain the type of physiology required for long term residence in this environment. In the case of *B. melitensis*, however, it is possible that the iron acquisition system which appears to be absent in *B. abortus* can compensate for loss of BacA function. Clearly, a

lot more investigation will be required before we are able to definitively establish the function of the *Brucella bacA* gene product.

III. *B. abortus* Tn*5* mutants which display nutritional defects *in vitro* and attenuation in cultured murine macrophages will be useful for assessing the nutritional requirements of the brucellae during residence in the host.

There is considerable evidence that the brucellae encounter nutritional deprivation during their long term residence in host macrophages (Crawford et al., 1996; Robertson and Roop, 1999). The nature of the nutrients available to these bacteria in the phagosomal compartment is also presently unknown. Similarly, little is known regarding the identity of the metabolic pathways in *Brucella* which are necessary for survival in this environment. Consequently, the identification of *B. abortus* mutants with well-defined nutritional and metabolic defects will provide useful reagents for evaluating the nutrient status of the phagosomal compartment within which the brucellae reside, as well as the metabolic versatility required by these bacteria for appropriate adaptation to this environment. Depending upon the extent and stability of the attenuation of these mutants when they are evaluated in mice, some of them may be useful as vaccine candidates. Bacterial mutants with well defined nutritional defects have been employed for this purpose for a variety of bacteria including *Salmonella typhimurium* (Curtiss and Kelly, 1987) and *Mycobacterium tuberculosis* (Hondalus et al., 2000). More importantly, the *B. melitensis purE* mutant presently being evaluated by investigators at Walter Reed as a vaccine candidate has a well defined defect in purine biosynthesis which leads to predictable and stable attenuation in BALB/c mice (Crawford et al., 1996), goats (Cheville et al., 1996) and primates (Borschel et al., 1999).

IV. Indirect experimental evidence suggests that the *Brucella* spp. possess an RpoS homolog which regulates stationary phase gene expression.

Previous studies in our laboratory have established that the *B. abortus hfq* gene product, an RNA binding protein known as host factor I, or HF-I, is required for stationary phase physiology and virulence (Roop and Robertson, 1999). In *E. coli* and *S. typhimurium*, HF-I is also required for stationary phase physiology because this protein facilitates the optimal translation of the mRNA encoding the stationary phase specific σ factor RpoS (Muffler et al., 1997). Because the phenotype of the *B. abortus hfq* mutant so closely resembles that of *E. coli* and *S. typhimurium hfq* and *rpoS* mutants, we have proposed that *B. abortus* possesses a functional RpoS homolog which is required for stationary phase gene expression, and that efficient expression of *rpoS* in *B. abortus* is dependent upon the function of HF-I (Robertson and Roop, 1999). To date, however, no *rpoS* homolog has been identified in any of the α–proteobacteria, let alone in the *Brucella* spp. Thus, our recent discovery of a potential *rpoS* homolog in *S. meliloti* provides us with an important tool for identifying the *rpoS* homolog in *Brucella*, if it exists. Once this gene (or its functional homolog) is identified, then genetic approaches can be used to identify and characterize the HF-I and RpoS regulated genes which contribute to virulence in the host.

V. C57BL6 NADPH oxidase knockout mice provide a useful tool for examining the importance of the oxidative killing pathways of host phagocytes in protective immunity against *Brucella* infections.

Numerous studies employing macrophages and neutrophils taken from a variety of animals have shown that oxidative killing is the primary mechanism by which host phagocytes control intracellular replication of the brucellae (Baldwin and Roop, 1999). In contrast, some studies employing *Brucella* mutants with well-defined defects in their oxidative defenses have raised questions regarding the importance of ROI-mediated killing by host macrophages in protective immunity against brucellosis (Grippe et al., 1999; Latimer et al., 1992; Tatum et al., 1992). For this reason, studies which employ NADPH oxidase mice and neutrophils and macrophages obtained from these animals will be important for better defining the importance of the oxidative killing pathways of host macrophages in protective immunity against *Brucella* infections.

VI. The apparent incompatibility of the *htrA* and *ctpA* mutations in *B. abortus* 2308 may provide insight into the physiologic functions of the corresponding gene products.

Biochemical and genetic evidence indicates that the HtrA and CtpA (also know as Prc, Tsp or Re) proteases function in the bacterial periplasm as stress response proteases which remove damaged proteins before they reach toxic levels (Lipinska et al., 1990; Hara et al., 1991). Notably, both of these proteases appear to be able to remove oxidatively damaged proteins and thereby help protect intracellular pathogens such as *S. typhimurium* (Bäumler et al., 1994) and *B. abortus* (Phillips and Roop, 1999) from ROI-mediated killing by host phagocytes. Because of the apparent overlap in the biological function of these proteases, it has been postulated that in some cases, one of these proteases may be able to compensate for the loss of the other. Evaluation of double mutants for synthetic enhancement of phenotype is a genetic strategy that is often used to determine whether or not two gene products have overlapping biological functions (Guarente 1993). In this regard, the apparent lethality of the combination of the *htrA* and *ctpA* mutations in *B. abortus* would certainly be consistent with overlapping biological function of the corresponding gene products, as either mutation alone does not impart an overly severe phenotype on the resulting mutant. Thus, it will be important to further analyze the compatibility or incompatibility of these mutations in *B. abortus* and *B. melitensis*, and if *htrA ctpA* double mutants can be constructed to evaluate these mutants for synthetic enhancement of phenotype.

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APPENDIX A. FIGURES AND TABLES FOR THE ANNUAL REPORT.

Figure 1. Intracellular survival and replication of *B. abortus* 2308, MEK6 (2308 *katE*) and MEK6 (pMEK21) in cultured murine resident peritoneal macrophages following opsonization with *Brucella*-specific hyperimmune mouse serum.

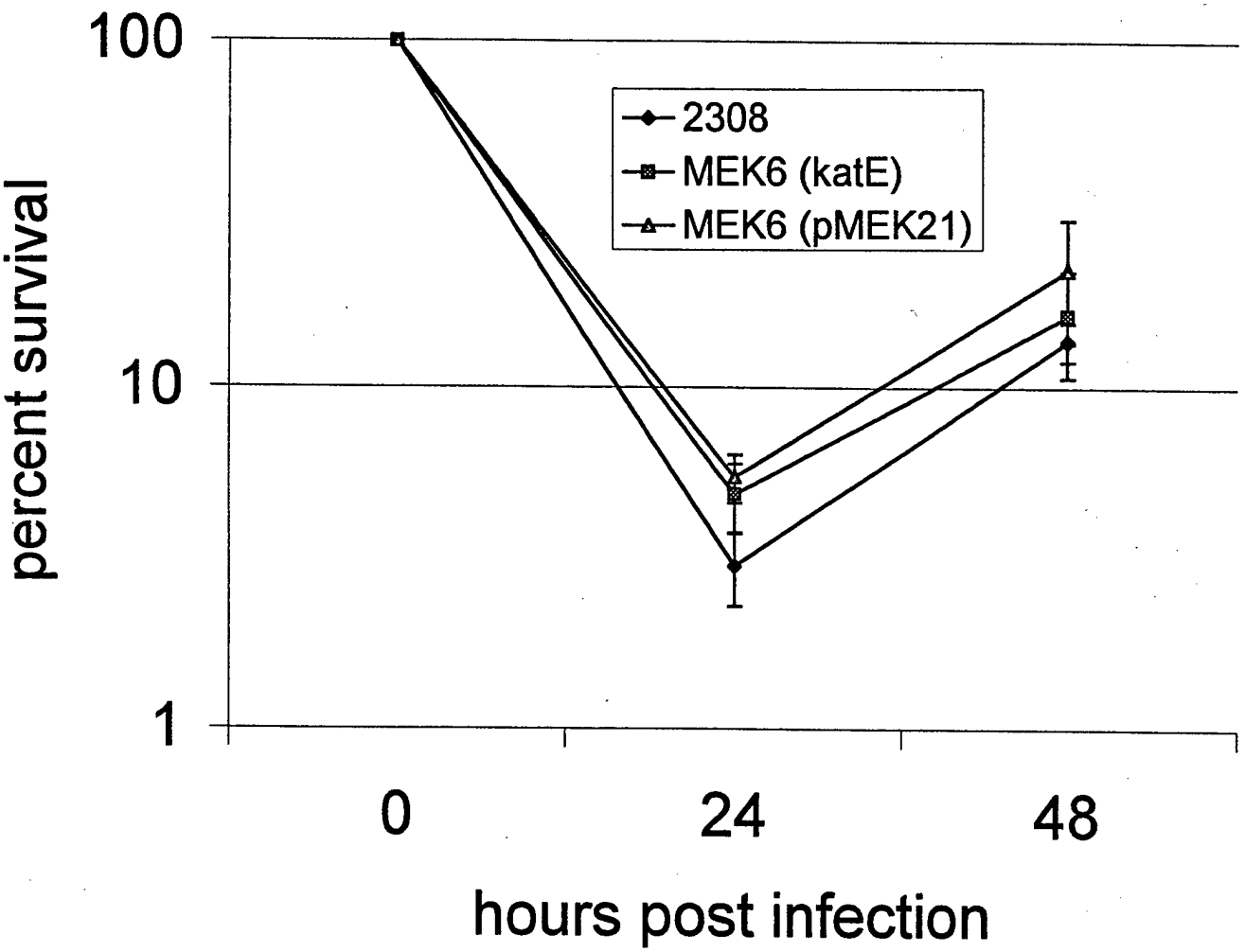

Figure 2. Sensitivity of *B. abortus* 2308, MEK6 (2308 *katE*) and MEK6 (pMEK21) to killing by H_2O_2 in disk sensitivity assays.

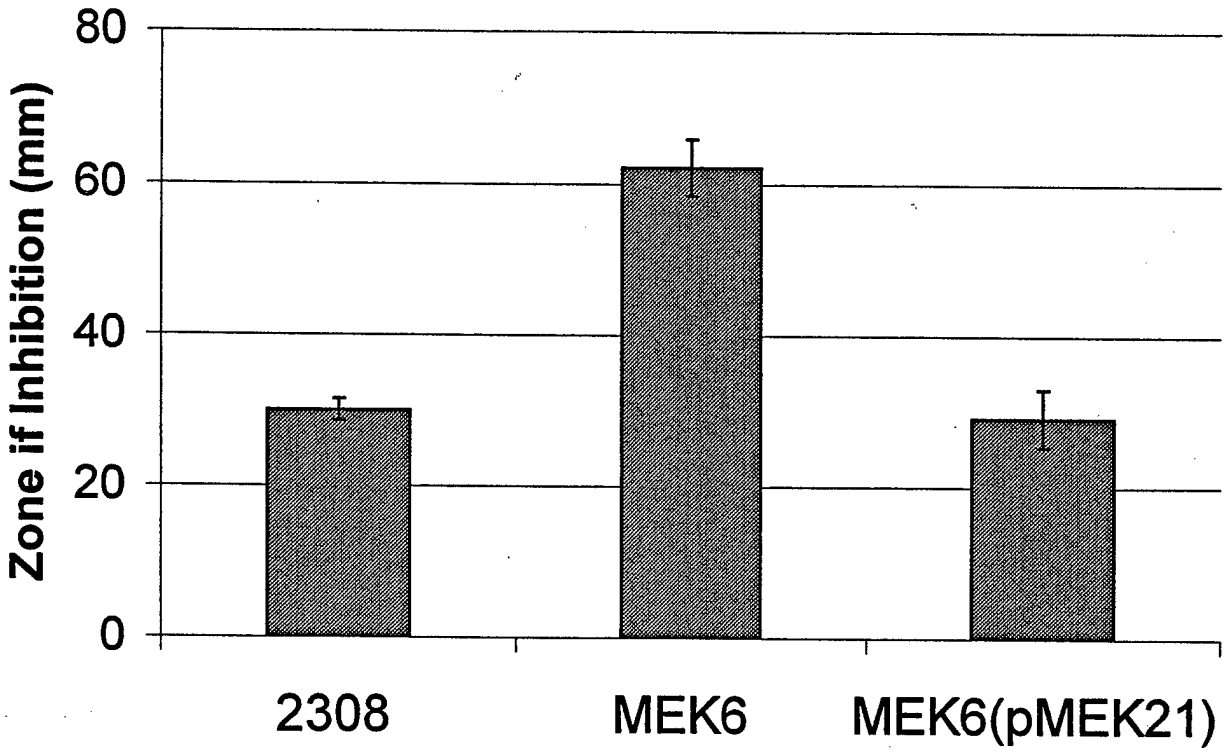

Figure 3. Spleen colonization profiles of *B. abortus* 2308, KL7 (2308 *bacA*) and KL74.3 (KL7 with the wild type *bacA* restored) in experimentally infected BALB/c mice.

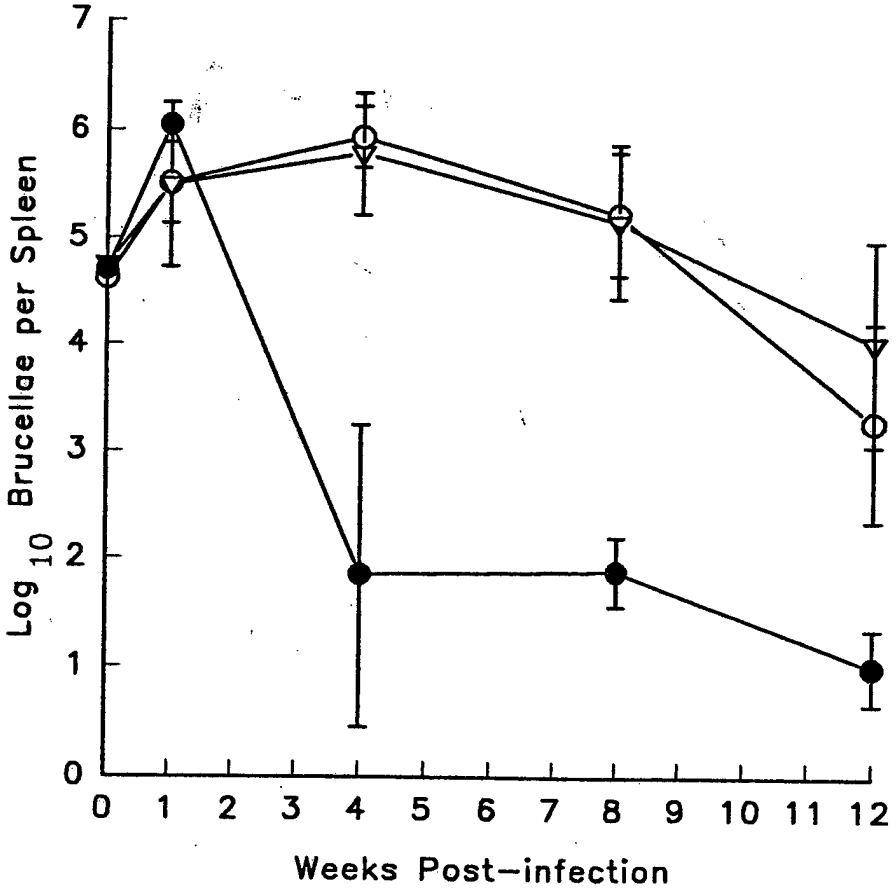

Figure 4. Intracellular survival and replication of *B. abortus* 2308, KL7 (2308 *bacA*) and KL74.3 (KL7 with the wild type *bacA* restored) in cultured resident peritoneal macrophages following opsonization with *Brucella*-specific hyperimmune mouse serum.

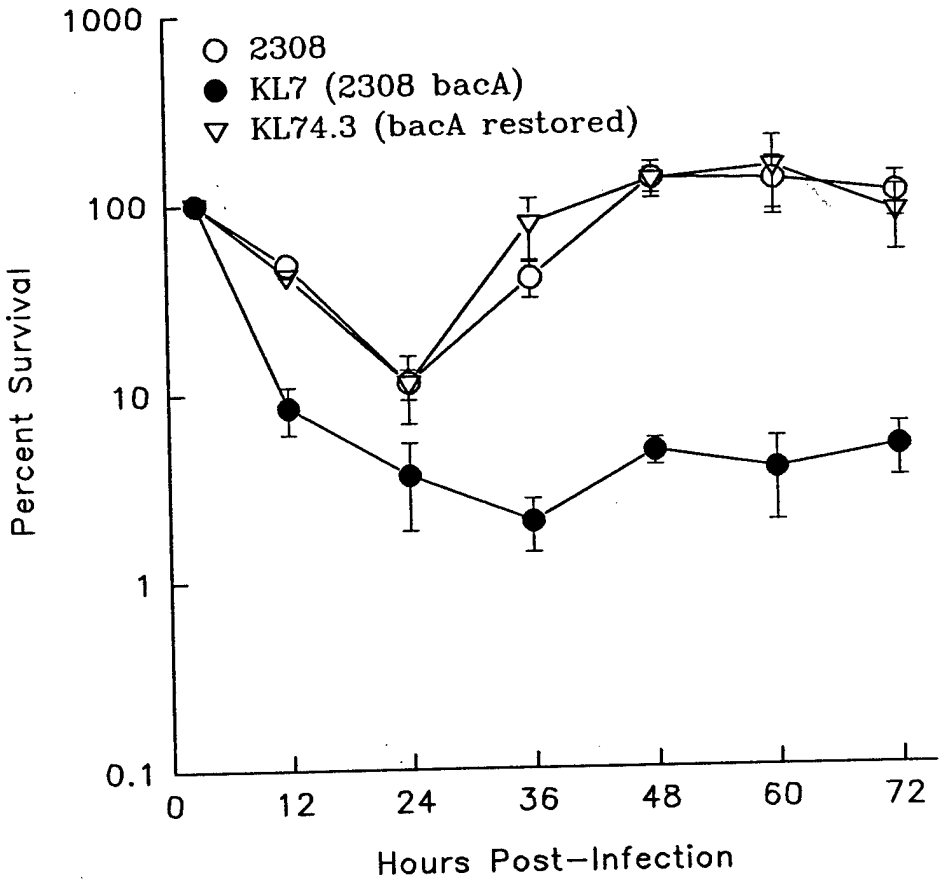

Figure 5. Intracellular survival and replication of *B. abortus* 2308, KL7 (2308 *bacA*) and KL74.3 (KL7 with the wild type *bacA* restored) in cultured resident peritoneal macrophages in the absence of opsonization.

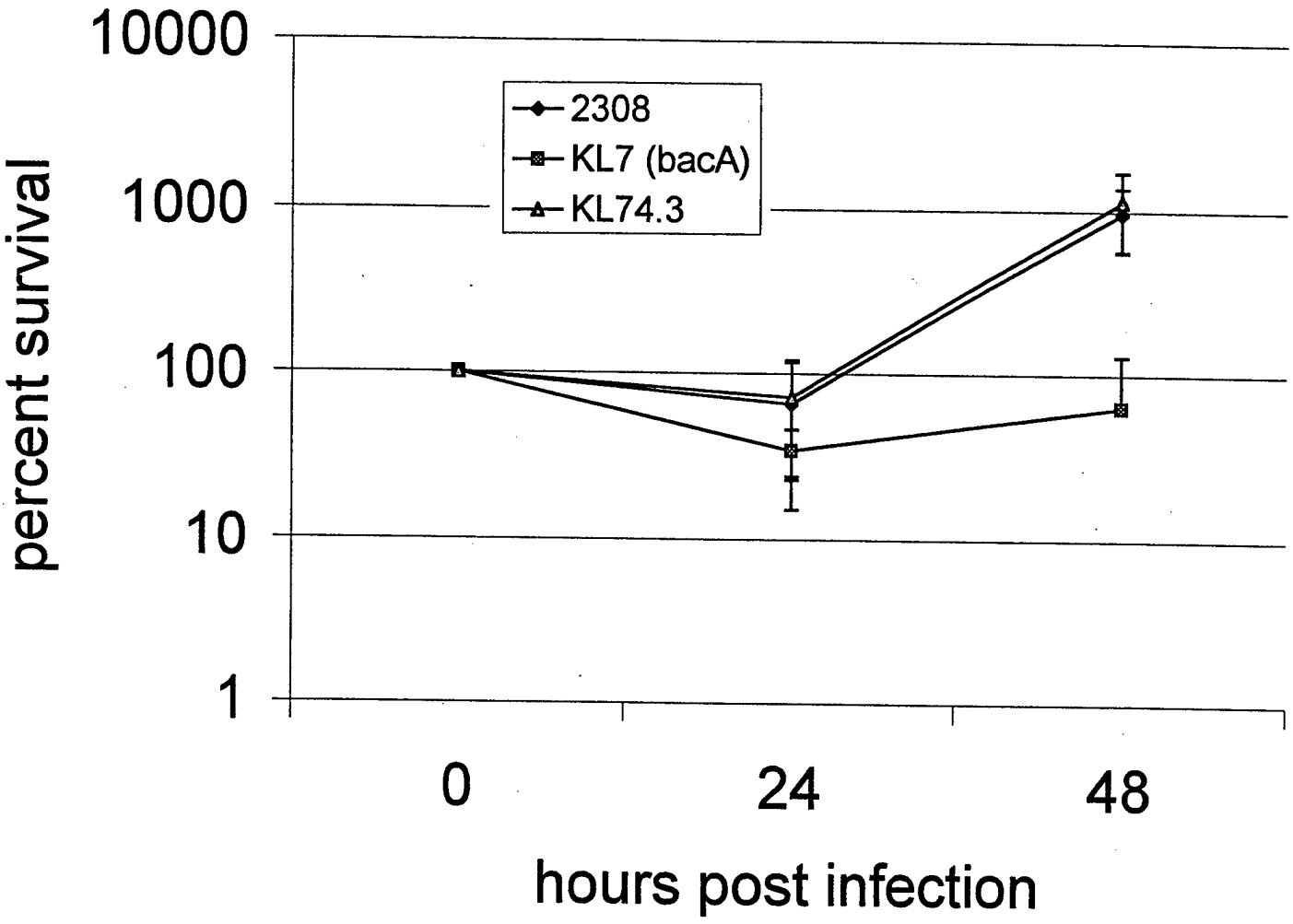

Figure 6. Growth of *B. abortus* 2308, KL7 (2308 *bacA*) and KL74.3 (KL7 with the wild type *bacA* restored) in brucella broth.

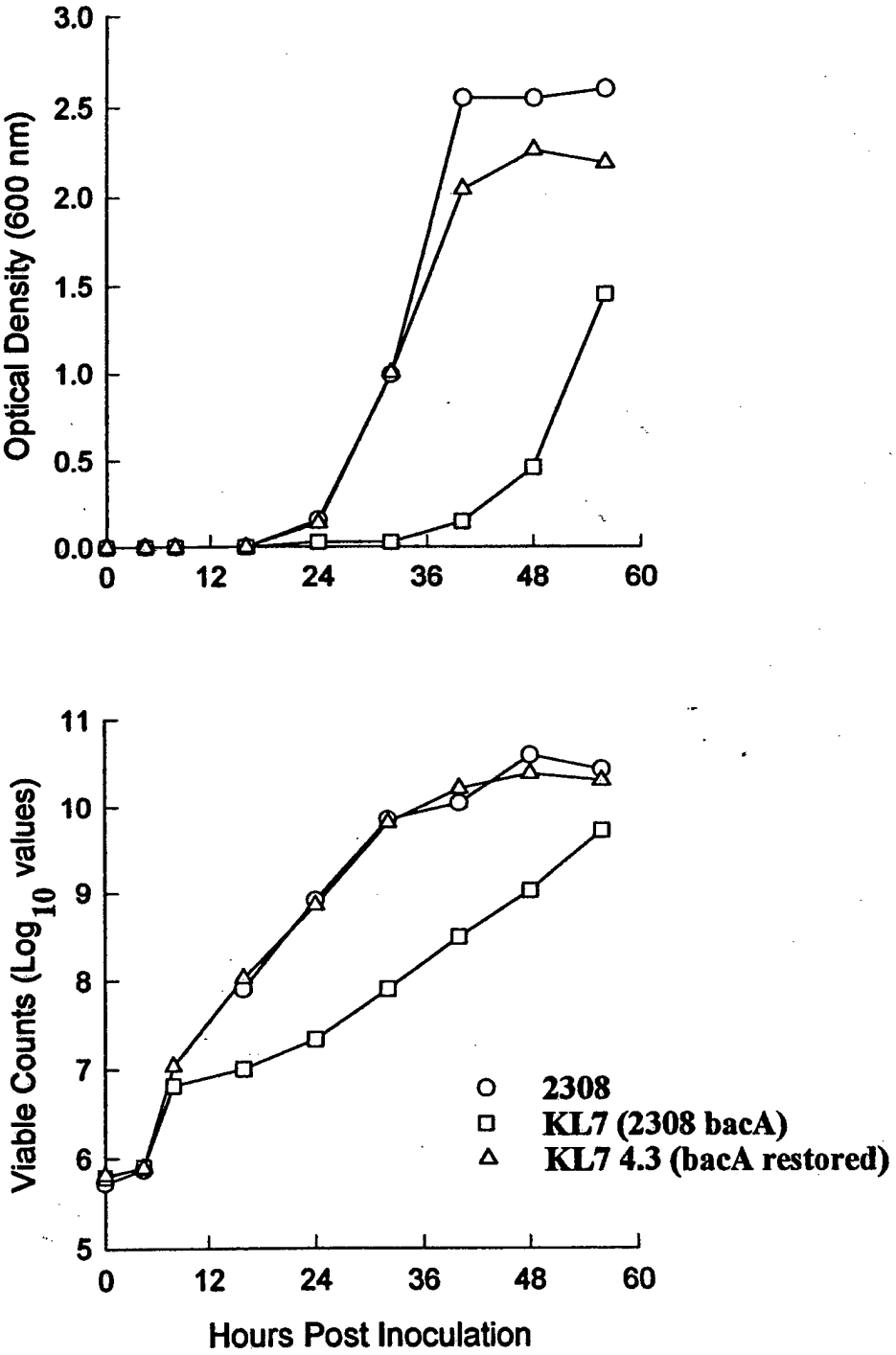

Figure 7. Spleen colonization profiles of *B. melitensis* 16M and KL20 (16M *bacA*) in experimentally infected BALB/c mice.

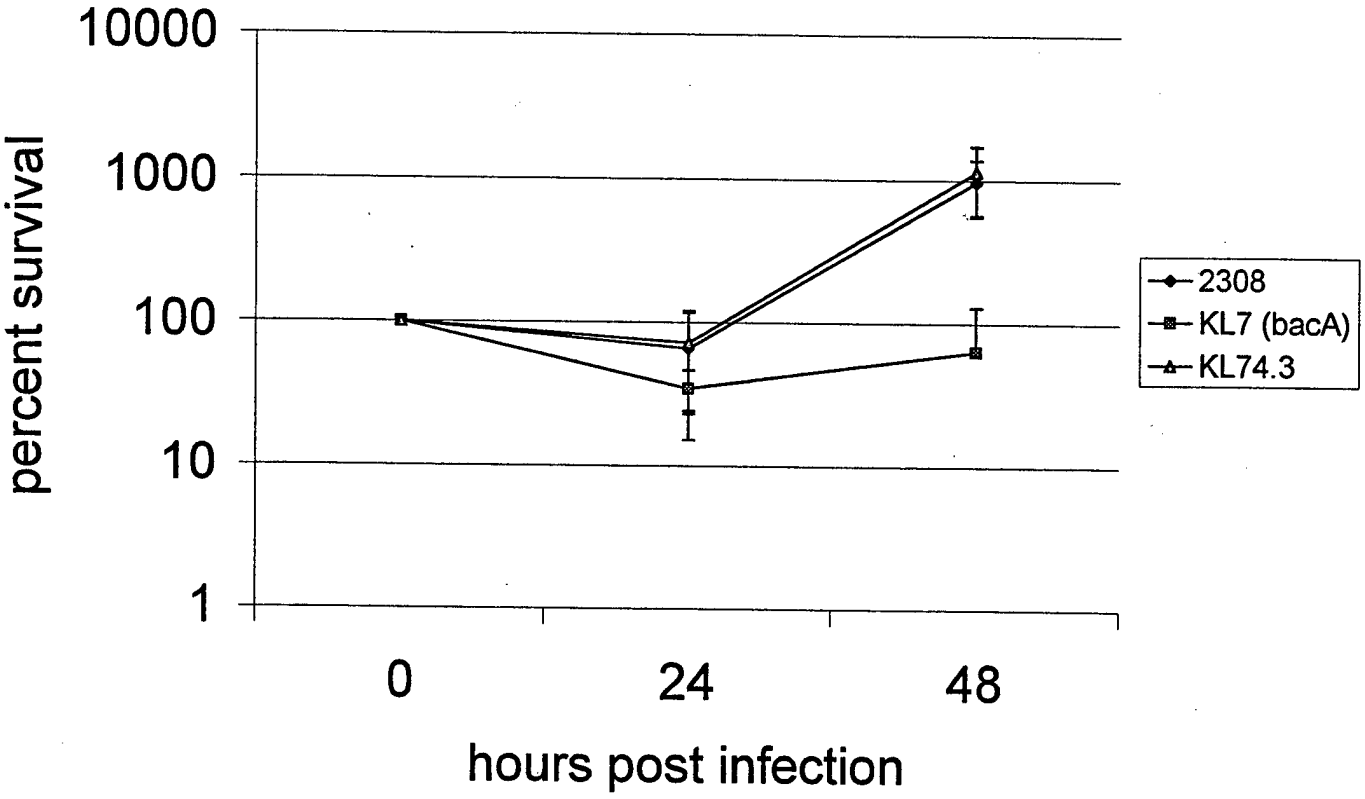

Figure 8. Intracellular survival and replication of *B. abortus* 2308 and selected Tn*5* mutants in cultured murine resident peritoneal macrophages following opsonization with *Brucella*-specific hyperimmune mouse serum.

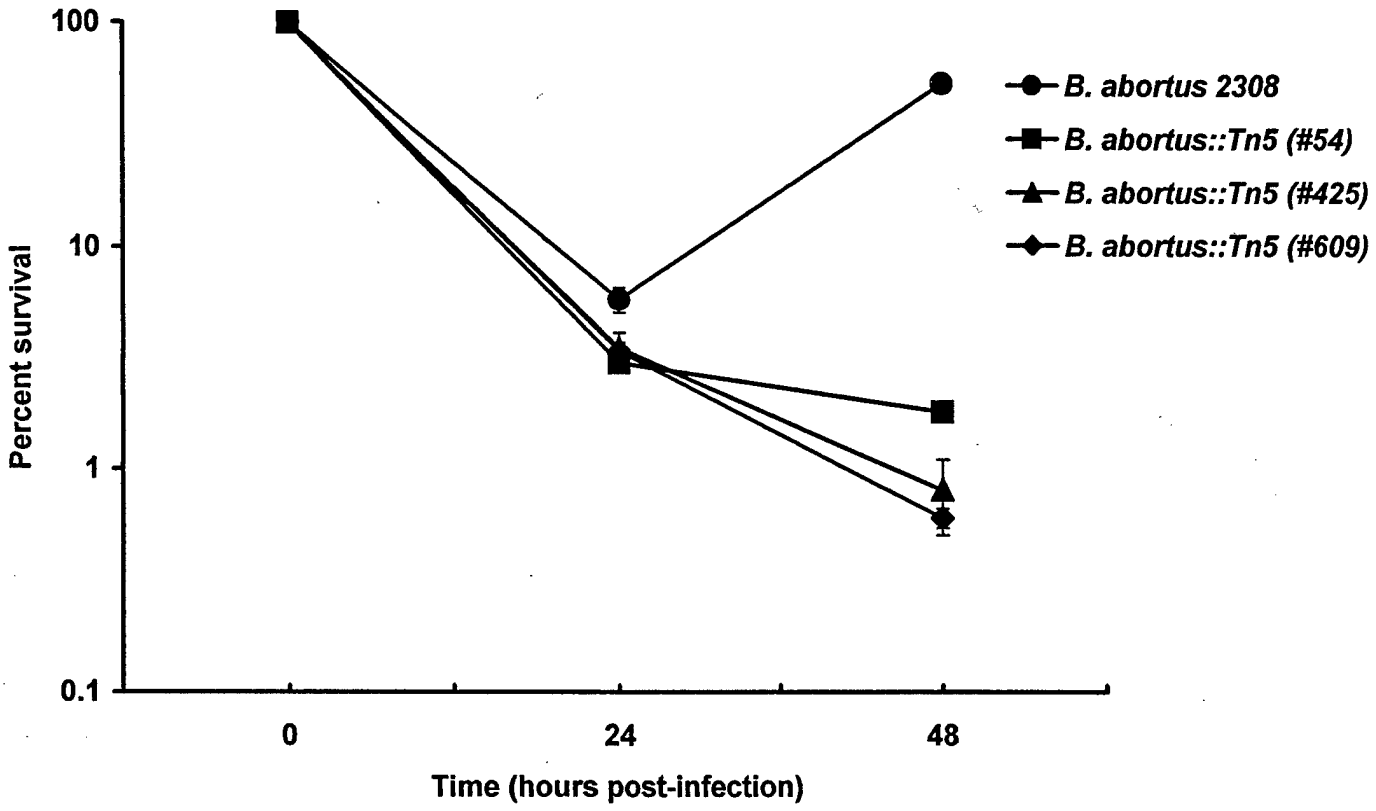

Figure 9. Spleen colonization profiles of *B. abortus* 2308 in C57BL6 NADPH oxidase knockout (*cybb*) mice and C57BL6 wild type mice.

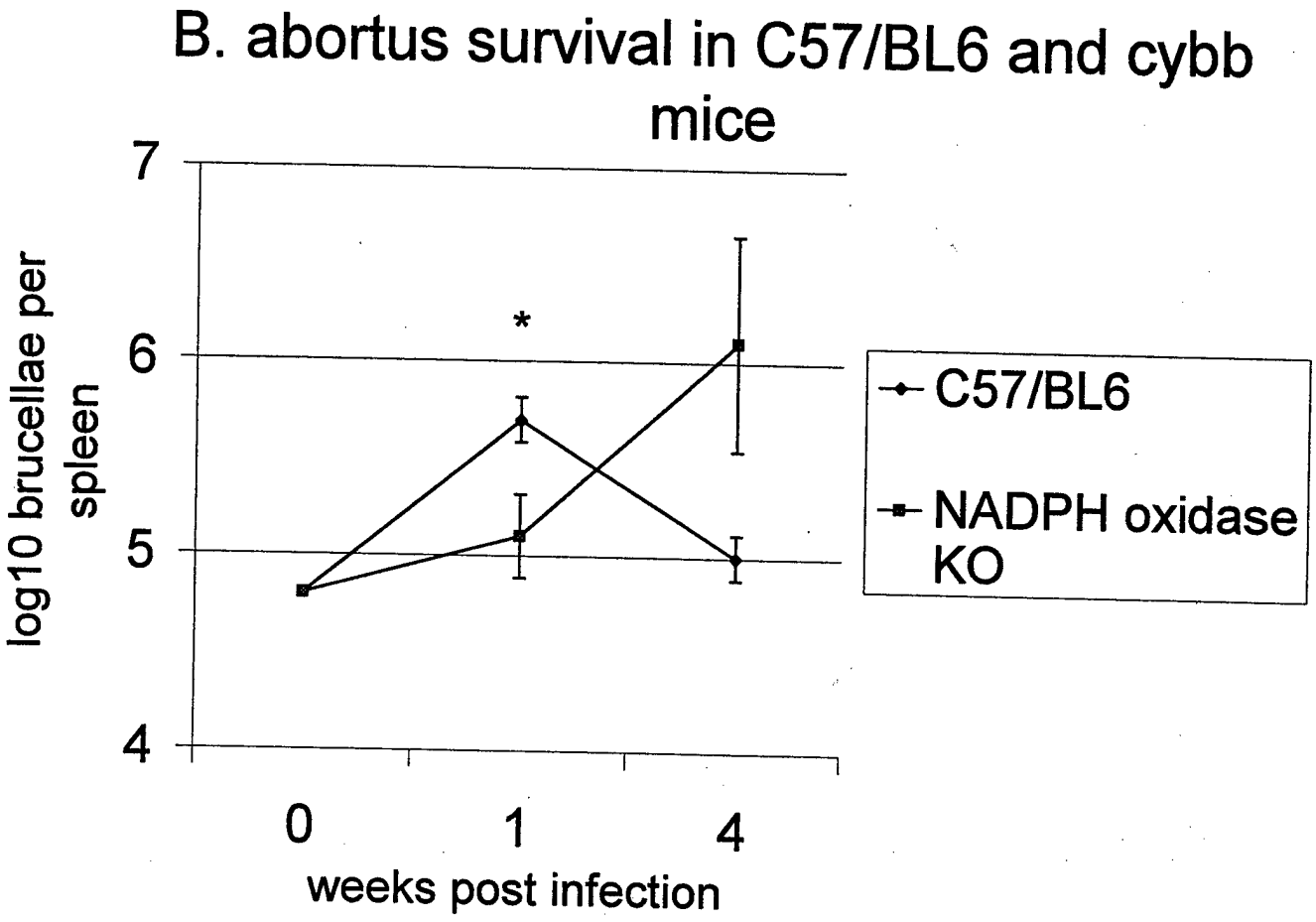

Figure 10. Intracellular survival and replication of *B. abortus* 2308 and VKG12 (2308 *ctpA*) in cultured murine resident peritoneal macrophages following opsonization with *Brucella*-specific hyperimmune mouse serum.